J Ind Microbiol Biotechnol (2003) 30: 216–219
DOI 10.1007/s10295-003-0041-5

ORIGINAL PAPER

Susana Delgado · Baltasar Mayo

Development of *Lactobacillus plantarum* LL441 and its plasmid-cured derivatives in cheese

Received: 10 June 2002 / Accepted: 12 February 2003 / Published online: 2 April 2003

Abstract A wild *Lactobacillus plantarum* strain and two of its plasmid-cured derivatives were separately used as adjunct cultures in the manufacture of a Gouda-like traditional Spanish cheese. The wild strain, LL441, harbours seven plasmids and produces a lantibiotic-like bacteriocin. The LL441-B2 derivative has lost plasmids of 40 and 80 kb and the bacteriocin-producing capability. The LL441-B11 derivative has lost in addition a 70 kb plasmid encoding active α- and β-galactosidases. All three strains could be used as adjunct cultures as none of the technological and biochemical parameters of the cheeses was affected. Both the wild-type and the two derivatives were recovered from experimental cheeses up to 30 days after manufacture at similar rates of nearly 20%. Thus, the phenotypic traits under examination were not essential for *L. plantarum* to grow into the cheese matrix.

Keywords NSLAB · *Lactobacillus plantarum* · Bacteriocin · β-Galactosidase · Adjunct cultures

Introduction

Mesophilic lactobacilli constitute the majority of non-starter lactic acid bacteria (NSLAB) in most ripened varieties of cheese. The dominant NSLAB species can be either homofermentative (*Lactobacillus plantarum*, *L. casei*, *L. paracasei* and *L. rhamnosus*) or heterofermentative (*L. brevis* and *L. fermentum*) [3, 4, 13]. The technological role played by these bacteria in the maturation of cheeses is not well understood. They all display diverse enzymatic activities that might accelerate or improve the development of cheese flavour during aging [7, 10, 11, 14]. However, heterofermentative lactobacilli have frequently been associated with undesirable flavours and other defects [10, 12]. The beneficial effects of adjunct cultures could also be based upon direct or indirect competition with undesirable bacteria [7, 12].

Mesophilic lactobacilli are capable of growing to high cell densities under the restrictive conditions of the curd (low E_h, low pH, limiting carbohydrates). Typical numbers range from 10^7 to 10^9 cfu g^{-1} within a few weeks post-manufacture [3]. Possible substrates for growth of NSLAB include residual lactose, citrate, fatty acids, glycerol, lactate, amino acids, nucleic acids and lysed starter cells [7]. Several authors have reported that the growth rate of NSLAB decreases as the fat content of the cheese is reduced [7, 11], which suggests that glycoproteins associated with the milk-fat globule membrane could be the sugar supplier. Moreover, most lactobacilli species possess glycoside hydrolases (α- and β-galactosidases, α- and β-galactosaminidases, *N*-acetyl-α-D-neuraminidase, etc.) able to release sugars from glycoproteins [16].

In this paper we report on the use of a *L. plantarum* strain and two of its plasmid-cured derivatives as adjunct cultures in cheese. The wild strain (LL441) harbours seven plasmids, produces a lantibiotic-like bacteriocin [9] and possesses functional copies of plasmid-encoded α- and β-galactosidase genes [6]. The LL441-B2 derivative has lost the bacteriocin-producing ability, while LL441-B11 has lost the bacteriocin and both plasmid-encoded galactosidases and, consequently, the capacity to ferment raffinose and melibiose [6]. Their suitability as adjunct cultures and their capability to multiply in cheese were investigated.

Materials and methods

Bacterial strains, media and culture conditions

L. plantarum cells were grown statically at 30°C in MRS broth (Biokar Diagnostics, Beauvois, France). To prepare adjunct cultures, cells were grown in MRS, then washed twice with a saline solution (0.9%).

S. Delgado · B. Mayo (✉)
Carretera de Infiesto s/n,
Instituto de Productos Lácteos de Asturias,
33300, Villaviciosa, Asturias, Spain
E-mail: baltasar_mayo@ipla.csic.es
Tel.: +34-985-892131
Fax: +34-985-892233

Manufacture and sampling of cheese

Four batches of Peñamellera cheese were manufactured from pasteurised cow's milk according to traditional technology [8], with Ezal MA011 (Texel, France) as a starter. Samples were taken according to standard procedures [2].

Biochemical and microbiological analyses

Some biochemical and microbiological parameters were analysed in milk, curd and cheeses of 3, 7, 15 and 30 days. Total solids, fat, NaCl, pH, titratable acidity, protein, total nitrogen, non-casein nitrogen and non-protein nitrogen were determined following standard methods [2].

For microbial analysis, 10 g of each cheese sample was homogenized in 90 ml 2% (w/v) sterilized sodium citrate at 45°C for 1 min in a Colworth Stomacher 400 (Seward, London, UK). Ten-fold dilutions were prepared in sterile 0.1% peptone water and plated on specific medium in duplicate.

Total bacterial counts

Total bacterial counts were determined on plate count agar (PCA; Biokar) containing 10 g l^{-1} skimmed milk powder (Oxoid, Basingstoke, UK) after incubation at 30°C for 72 h.

Lactococci

Lactococci were enumerated on M17 agar (Biokar). The medium was supplemented with 40 μg ml^{-1} nalidixic acid (Sigma, St. Louis, Mo.) for counts involving milk and curd samples. Incubation was at 30°C for 48 h.

Lactobacilli

Lactobacilli were counted on MRS agar (Biokar), pH 5.4, after 72 h of incubation at 30°C in anaerobic jars with an enriched CO_2 atmosphere (Anaerocult C; Merck, Darmstadt, Germany).

Enterobacteriaceae and coliforms

Violet red bile lactose (VRBL) agar (Biokar) was used to enumerate both *Enterobacteriaceae* ssp. and coliforms, using the pour-plate and overlay technique. Incubations were for 24–48 h at 30°C.

Enterococci

Enterococci were scored after 24 h of incubation at 44°C in KF agar (Adsa-Micro, Barcelona, Spain) containing 10 g l^{-1} triphenyltetrazolium chloride.

Staphylococci

Dilutions were plated on Baird-Parker agar (Biokar) and incubated for 24 h at 37°C. Black colonies with or without egg yolk clearing were recorded.

Yeasts and moulds

Dilutions of milk and cheese samples were plated on chloramphenicol glucose agar (CGA) (Merck) and incubated for 5 days at 25°C.

Carbohydrate fermentation profiles

Carbohydrate fermentation profiles were determined by the API 50 CHL system (bioMérieux, Montalieu-Vercieu, France) according to the manufacturer's instructions.

Antimicrobial activity

Cross-antagonistic activities were assayed by an agar spot test [9], using *Lactobacillus sakei* CECT 906 (Spanish Type Culture Collection, University of Valencia, Burjasot, Spain) as an indicator strain.

Plasmid typing

Plasmid DNA from *Lactobacillus* ssp. strains was isolated by the method of Anderson and McKay [1] and electrophoresed in 0.75% agarose gels in 89 mol l^{-1} TBE.

Results and discussion

The development of the main microbial populations in milk, curd and experimental cheeses during manufacture and ripening of the four batches of Peñamellera cheese are shown in Table 1. Milk was not of high quality; counts of total aerobic bacteria after pasteurisation were close to 4.5×10^4 cells ml^{-1}. *Enterobacteriaceae* and coliforms were among the dominant bacteria in pasteurised milk ($\sim3.5\times10^3$ cells ml^{-1}). Lactobacilli counts in milk were around 2.0×10^2 ml^{-1}. In experimental cheeses, adjunct cultures were added at around 1.0×10^5 cells ml^{-1}. Lactococci, present at 2.5×10^2 cells ml^{-1} in milk, rose to more than 5.0×10^8 cells ml^{-1} in curd after addition of the starter.

The greatest number of bacteria in all Peñamellera cheeses was seen at day 3. Similar data have been described for traditional cheese elsewhere [5]. Total counts in PCA coincided with numbers of lactococci throughout the ripening stage (Table 1). After lactococci, lactobacilli (NSLAB) formed the most numerous population, with numbers reaching their maximum between days 3 and 7, depending on the batch. The development of NSLAB in all four batches was similar and differences were not statistically significant (not shown). The development of all other populations examined (coliforms, enterococci, staphylococci, and yeasts and moulds) was similar for all batches. However, in trials with adjunct cultures, they reached maximum numbers later and the final values were somewhat lower than in the control cheese.

The NSLAB composition of control and experimental batches was followed in several ways. Representative strains were subjected to the API system to obtain their carbohydrate fermentation profiles and to check for melibiose and raffinose fermentation. Since plasmid profiles of all strains under assay were known [6], plasmid content was frequently used to monitor NSLAB components. Bacteriocin production in solid medium was used to follow up the evolution of *L. plantarum*

Table 1 Evolution of counts (log_{10} cfu g^{-1}) of the main microbial populations during manufacture and ripening of the four batches of Peñamellera cheese. *NSLAB* Non-starter lactic acid bacteria

Microbial groups	Trial[a]	Milk[b]	Curd	Cheese sample (ripening time)			
				3 days	7 days	15 days	30 days
Total aerobic counts	C	4.20	8.50	9.43	9.28	9.18	8.98
	1		8.70	9.08	9.02	9.04	8.80
	2		8.88	9.32	9.43	9.15	8.90
	3		8.52	9.48	9.40	9.02	8.78
Lactococci	C	2.47	8.52	9.45	9.20	9.18	8.70
	1		8.68	9.40	9.13	8.99	8.90
	2		8.70	9.40	9.32	9.15	8.92
	3		8.40	9.49	9.37	9.32	9.02
Lactobacilli (NSLAB)	C	2.00	3.21	8.07	8.25	7.98	8.04
	1		4.89	8.30	8.25	8.11	7.69
	2		4.94	7.57	8.18	7.94	7.27
	3		5.15	7.78	8.01	7.93	7.28
Enterococci	C	1.60	2.51	6.33	5.18	5.40	5.66
	1		2.63	5.60	5.36	4.78	5.67
	2		2.60	5.52	5.00	4.70	5.70
	3		2.87	5.45	5.04	4.84	5.70
Staphylococci	C	1.28	1.98	6.60	3.91	4.77	4.18
	1		2.04	3.34	3.78	4.68	4.18
	2		2.35	4.05	3.83	4.24	3.99
	3		2.53	3.86	4.25	4.10	4.29
Coliforms	C	2.40	3.92	6.60	5.20	5.60	4.33
	1		4.63	5.77	4.86	5.91	4.41
	2		4.13	5.15	5.18	5.77	4.24
	3		4.41	5.42	5.02	5.78	3.99
Yeast and moulds	C	1.70	2.46	6.43	5.49	5.60	5.43
	1		2.34	5.57	4.86	5.91	4.41
	2		2.36	5.25	5.72	5.38	5.89
	3		2.26	5.10	5.94	5.54	5.21

[a]*C* Control cheese, without any added adjunct culture; *1*, *2*, *3* experimental cheeses elaborated with *Lactobacillus plantarun* LL441 wild-type strain, *L. plantarum* LL441-B2, and *L. plantarum* LL441-B11 as adjunct cultures, respectively

[b]Average of the four samples from each trial

Table 2 Relevant biochemical parameters of milk, curd and experimental cheeses during ripening. *TS* Total solids, *NPN* non-protein nitrogen

Microbial groups	Trial[a]	Milk[b]	Curd	Cheese sample (ripening time)			
				3 days	7 days	15 days	30 days
pH	C	6.59	6.71	5.66	5.25	5.32	5.42
	1		6.68	5.37	5.26	5.22	5.27
	2		6.71	5.59	5.41	5.33	5.30
	3		6.64	5.26	5.15	5.17	5.15
Fat (%)	C	3.65	13.5	24.5	29.5	27.5	29.5
	1		16.0	25.5	26.0	28.0	29.5
	2		15.0	25.5	26.0	27.0	27.5
	3		15.0	24.0	25.0	27.0	28.5
NaCl (g/100 g)	C	0.12	1.02	1.28	1.07	1.91	1.02
	1		1.37	1.54	1.25	1.66	1.76
	2		1.25	1.65	1.04	1.06	1.78
	3		1.14	0.95	0.90	1.15	1.23
TS (%)	C	11.36	33.47	52.59	55.62	58.18	62.57
	1		35.98	54.40	57.20	57.20	61.93
	2		35.61	54.83	56.38	58.18	61.43
	3		36.16	51.74	54.54	57.94	62.01
Protein (%)	C	3.02	11.71	22.27	23.44	24.61	26.75
	1		14.70	22.46	23.77	23.84	25.07
	2		14.37	22.78	23.47	24.39	25.11
	3		13.09	21.48	22.65	22.72	25.53
Total nitrogen (%)	C	0.473	1.184	3.491	3.675	3.859	4.193
	1		2.305	3.521	3.727	3.737	3.960
	2		2.252	3.572	3.679	3.824	3.936
	3		2.053	3.368	3.550	3.562	4.003
NPN (%)	C	0.021	0.046	0.134	0.162	0.181	0.235
	1		0.042	0.151	0.126	0.165	0.189
	2		0.039	0.134	0.155	0.210	0.263
	3		0.032	0.148	0.157	0.200	0.191

[a]Trial conditions as in Table 1

LL441 in cheese [9]. Plasmid profiles of NSLAB from the control cheese were unrelated to those displayed by the adjunct strains, indicating that no cross-contamination occurred. Using LL441 as the starter adjunct, around 25% of the NSLAB isolates produced antimicrobial substances on day 3. All producing isolates proved to be LL441 after examination of their plasmid content. This strain was isolated as part of the dominant NSLAB of a traditional cheese [9]. Thus, it was not surprising that *L. plantarum* LL441 could multiply well in cheese. Nonetheless, other adventitious NSLAB lactobacilli multiplied as well and accounted for 75% of the total at this time. It was previously reported that the bacteriocin produced by LL441 is not active against *L. plantarum* and *L. casei* strains [9]. Therefore, this property, which is thought to be a factor of competition in certain habitats [15], is not a selective advantage against the commonest NSLAB species. Percentages of LL441 during ripening were 21% at day 7, 19% at day 15, and 20% at day 30. Thus, they were maintained at a similar level throughout the whole ripening period. Similar percentages of dominance were also seen for strains LL441-B2 (average 18%) and LL441-B11 (average 23%). This further demonstrated that bacteriocin production in LL441 was not implicated in dominance of this strain in the cheese environment. From these results, we concluded that the genetic material lost by the LL441 derivatives was neither involved in growth nor in maintenance of these strains in cheese. Thus, the plasmid-encoded α- and β-galactosidases (and the biochemical machinery to use raffinose and melibiose that was lost) were either bypassed or are not essential for growth in this medium.

The chemical and biochemical parameters of milk, curds and cheeses are given in Table 2. Small differences were detected among trials for some parameters. Fluctuations in salt content might be caused due to unequal distribution of salt (as the curd is manually salted) [5]. Fat, total solids, protein, total nitrogen and non-protein nitrogen contents were nearly identical in all samples. Differences similar to those in Table 2 were obtained with replicates of the same sample. Large differences between control and experimental trials were not expected, since the ripening period of the cheese (30 days) is too short for differences in proteolysis to be appreciated, as has been repeatedly reported [7, 8, 13]. The textural and sensory characteristics of all trials were evaluated by hedonistic analysis and no significant differences were observed between control and experimental cheeses (data not shown).

In conclusion, *L. plantarum* LL441 and its derivatives can be used with confidence as adjunct cultures, because they do not affect manufacture and ripening parameters of Peñamellera cheese. However, under the conditions used in this work (a high initial NSLAB population and a short ripening time), their use did not lead to detectable improvement of textural or flavour attributes.

Acknowledgements This work was supported by the Comisión Interministerial de Ciencia y Tecnología (CICYT), (Grant 97FD-0339). The authors thank the firm SCL Quesos de Peñamellera, which kindly offered its facilities for experimental trials. The skilful assistance of M.J. González is gratefully acknowledged.

References

1. Anderson DG, McKay LL (1983) Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl Environ Microbiol 46:549–552
2. AOAC (1984) William S (ed) Official Methods of Analysis of the Association of Official Analytical Chemist, 14th edn. AOAC, Arlington, Va.
3. Beresford TP, Fitzsimons NA, Brennan NL, Cogan TM (2001) Recent advances in cheese microbiology. Int Dairy J 11:259–274
4. Crow V, Curry B, Hayes M (2001) The ecology of non-starter lactic acid bacteria (NSLAB) and their use as adjuncts in New Zealand Cheddar. Int Dairy J 11:275–283
5. Estepar MJ, Sánchez MM, Alonso L, Mayo B (1999) Biochemical and microbiological characterization of Peñamellera cheese: analysis of its indigenous lactic acid bacteria. Int Dairy J 9:737–746
6. Fernández M, Margolles A, Suárez JE, Mayo B (1999) Duplication of the β-galactosidase gene in some *Lactobacillus plantarum* strains. Int Dairy J 48:113–123
7. Fox PF, McSweeney PLH, Lynch CM (1998) Significance of non-starter lactic acid bacteria in Cheddar cheese. Aust J Dairy Technol 53:83–89
8. Gómez MJ, Gaya P, Núñez M, Medina M (1996) Effect of *Lactobacillus plantarum* as adjunct starter on the flavour and texture of a semi-hard cheese made from pasteurized cow's milk. Lait 76:461–472
9. González B, Arca P, Mayo B, Suárez JE (1994) Detection, purification, and partial characterization of plantaricin C, a bacteriocin produced by a *Lactobacillus plantarum* strain of dairy origin. Appl Environ Microbiol 60:2158–2163
10. Khalid NM, Marth EH (1990) Lactobacilli, their enzymes and role in ripening and spoilage of cheese: a review. J Dairy Sci 73:2669–2684
11. Lane CN, Fox PF (1996) Contribution of starter and adjunct lactobacilli to proteolysis in Cheddar cheese during ripening. Int Dairy J 6:715–728
12. Martley FG, Crow VL (1993) Interactions between non-starter organisms during cheese manufacturing and ripening. Int Dairy J 3:461–483
13. McSweeney PLH, Fox PF, Lucey JA, Jordan KN, Cogan TM (1993) Contribution of the indigenous microflora to the maturation of Cheddar cheese. Int Dairy J 3:613–634
14. Peterson SD, Marshall RT, Heymann H (1990) Peptidase profiling of lactobacilli associated with Cheddar cheese and its application to identification and selection of strains for cheese-ripening studies. J Dairy Sci 73:1454–1464
15. Vuyst L de, Vandamme E (1994) Antimicrobial potential of lactic acid bacteria. In: de Vuyst L, Vandamme E (eds) Bacteriocins of lactic acid bacteria. Blackie, London, pp 91–142
16. Williams AG, Banks JM (1997) Proteolytic and other hydrolytic enzyme activities in non-starter lactic acid bacteria (NSLAB) isolated from Cheddar cheese manufactured in the United Kingdom. Int Dairy J 7:763–774